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A Non-invasive Method for Generating the Cyclic Loading-induced Intra-articular Cartilage Lesion Model of the Rat Knee

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TITLE:

A Non-invasive Method for Generating the Cyclic Loading-induced Intra-articular Cartilage Lesion Model of the Rat Knee

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KEYWORDS:

In vivo cyclic compression; Post-traumatic osteoarthritis; Cartilage degeneration; Rat model

SUMMARY:

Here, we present the cyclic loading-induced intra-articular cartilage lesion model of the rat knee, generated by 60 cyclic compressions over 20 N, resulting in damage to the femoral condylar cartilage in rats.

ABSTRACT:

The pathophysiology of primary osteoarthritis (OA) remains unclear. However, a specific subclassification of OA in relatively younger age groups is likely correlated with a history of articular cartilage damage and ligament avulsion. Surgical animal models of OA of the knee play an important role in understanding the onset and progression of post-traumatic OA and aid in the development of novel therapies for this disease. However, non-surgical models have been recently considered to avoid traumatic inflammation that could affect the evaluation of the intervention.

In this study, an intra-articular cartilage lesion rat model induced by *in vivo* cyclic compressive loading was developed, which allowed researchers to (1) determine the optimal magnitude, speed, and duration of load that could cause focal cartilage damage; (2) assess post-traumatic spatiotemporal pathological changes in chondrocyte vitality; and (3) evaluate the histological expression of destructive or protective molecules that are involved in the adaptation and repair

mechanisms against joint compressive loads. This report describes the experimental protocol for this novel cartilage lesion in a rat model.

INTRODUCTION:

Traditionally, anterior cruciate ligament (ACL) transection or destabilization of the medial meniscus has been considered optimal for investigating post-traumatic osteoarthritis (PTOA) in small animals. In recent years, non-invasive cyclic compression models have been used to study PTOA. This model was originally designed to investigate the cancellous bone response to mechanical loading[1] and was then modified as a non-surgical animal model for PTOA studies[2-6]. The rationale is to rupture the articular cartilage by applying a periodic external force, which triggers a series of inflammatory responses. However, this model has only been applied to mice, and the appropriate magnitude of loading on larger animals has not been discussed.

Another problem with the previous model is that the high-volume protocol included too many cycles, which caused excessive thickening of the subchondral bone, an unwanted side effect, in several samples[7]. Therefore, a novel method of cyclic compression with the appropriate magnitude for large animals and a lower loading side effect was developed[8]. The overall goal of the current article is to describe the protocol of the non-invasive cyclic compression model in rats and observe the representative results of cartilage degeneration. The current protocol would help readers interested in the application of the non-invasive cyclic compression model on rats.

PROTOCOL:

The protocol was approved by the Animal Research Committee of Kyoto University (approval number: Med kyo 17616).

1. Perform *in vivo* cyclic compression on the rat knee

1.1. Induce experimental animal anesthesia

1.1.1. Induce anesthesia in a 12-week-old Wistar rat (256.8 ± 8.7 g) by inhalation of 5% isoflurane solution in the anesthesia box.

1.1.2. Inject a mixture of three anesthetic agents[9], including medetomidine, midazolam, and butorphanol, at 2 mg/kg of the rat body weight, and shave the area around the right knee joint. Confirm sufficient anesthetization by testing for the toe-pinch response with forceps, ensuring that the rat responds without any struggle after anesthesia while pinching.

1.2. Mount the anesthetized rat on the fixation device.

1.2.1. Place the anesthetized rat lying on their belly on the baseplate (**Figure 1**), with the right knee attached to a small piece of resin with a concave groove. Place the right hind limb in the hip extension, knee flexion, and ankle extension positions, with the knee flexed at approximately 140°. Accommodate the heel of the rat on the wedge-shaped groove on the movable fixture.

1.2.2. Move the fixation device to the stress/tensile testing instrument (see the **Table of Materials**). After ensuring that there are no contacts with the load cell, open the stress/tensile testing instrument control software (**Table of Materials**) and click on the **Calibration** button. After calibration, attach the top of the frame to the load cell carefully. To keep the knee joint closely attached to the frame, turn on the rotary knob on the movable main operational panel slowly until the pre-load reaches 5 N.

1.3. Build a loading method and set up the compressive test.

1.3.1. On the **Main menu**, click on **Create a new method** | **System** label. Set **Test Mode** to **Cycle**, and **Test Type** to **Compression**. Click on the **Sensor** label and select the **Test** tab to check that the limit is within 60 N. In addition, select the **Stroke** tab and check that the limit is within 500 mm.

NOTE: The above step will stop the operation immediately if there is a large displacement on the stress point.

1.3.2. Under the **Testing control** label, select **Origin of growth** to start the main program with 0.3%/full scale. Of the four sections in a loading cycle, set the **Stroke speed in control** in the 1st and 3rd sections to 1 mm/s. Set the **Maximum testing force** in the 2nd section to 20 N, and the **Minimum testing force** in the 4th section to 5 N. Set "**the Duration of hold**" to 0.5 s for the peak load and 10 s for the minimum load (**Figure 2**).

NOTE: As this step defines every cycle, ensure that the joint surfaces are in contact with each other and are moving at a reasonable speed and that the motion is maintained.

1.3.3. In the **Pre-load** tab at the bottom of the page, ensure that **On** is checked, the **Speed of deflection removal** is set to 100 mm/min, and **maximum force** is 5 N. In the **Specimen** label, set the **Material** as **Metal**.

NOTE: These detailed settings may be specific for each manufacturer.

1.3.4. In the **Main menu**, under the **Select method and test** section, select the method that was just built, and click on **Start** to begin the test.

NOTE: The table at the bottom shows the actual measurements of the peak load and displacement.

1.3.5. Set the number of cycles to 60.

NOTE: The entire loading session includes 60 cycles, which lasts approximately 12 min. In the control group, rats underwent 5 N pre-loading for 12 min pre-load under the same conditions.

1.4. After loading, return the rat to its cage and maintain a 12-12 h light-dark schedule in a cage

with sufficient space and food *ad libitum* until it is sacrificed by the injection of an overdose of anesthetic agents or carbon dioxide inhalation for analysis (1 h–8 weeks).

REPRESENTATIVE RESULTS:

A representative result of the short-term changes (1 h and 12 h) in chondrocyte viability in samples subjected to 20 N cyclic loading was obtained. As shown in **Figure 3**, the number of dead chondrocytes (red fluorescence) increased at 12 h post-trauma. Conversely, the number of living chondrocytes (green fluorescence) continued to decrease, with some samples containing no live chondrocytes in the affected area.

Histology showed that the articular cartilage of the rat knees that underwent 20 N dynamic loading was damaged, and one focal lesion zone was confirmed in the lateral femoral condyle in all the samples (**Figure 4**). However, the lesion size did not progressively increase during the 8-week observational period. The border that corresponded to the interface of the lesion and the unaffected cartilage could be observed in the affected area.

FIGURE LEGENDS:

Figure 1: The fixation device consists of a baseplate and a fixation apparatus. The base plate (length: 27.5 cm, width: 13 cm) has a resin concave groove (length: 0.8 cm, width: 0.4 cm) on the posterior side to accommodate the flexed knee joint of the rat. The fixation apparatus has a wedge-shaped groove (groove width: 1.5 cm, groove depth: 1 cm) that accommodates the rat's heel, which is nested in the baseplate between two metal bars. The top of the fixation apparatus will be in direct contact with the load cell of the stress/tensile testing instrument.

Figure 2: Load profile for one cycle of loading.

Figure 3: Spatiotemporal assessment of chondrocyte viability in the lesion area. After sacrifice, the knee joint was dissected and separated using small forceps and scissors. Solutions of calcein AM and EthD-1 stains were prepared by diluting the original kit (**Table of Materials**) at 1:500 and 1:4,000 in 5 mL of PBS, respectively. The samples were incubated for 20 min at room temperature. Control samples were immersed in PBS under the same conditions. Fluorescence images were obtained using a fluorescence microscope (**Table of Materials**) using fluorescein isothiocyanate (495 nm/519 nm) and propidium Iodide (535 nm/617 nm) channels. The vital chondrocytes displayed green fluorescence, whereas dead cells fluoresced red. Compared to the chondrocytes in control samples (**A**), the number of dead chondrocytes on the loaded rat knee was increased at 1 h (**B**) and occupied most of the area in the affected region at 12 h (**C**). Green and red fluorescence represent the regions of the live and dead chondrocytes, respectively. Scale bars = 100 µm. Abbreviations: calcein AM = calcein acetoxymethyl ester; EthD-1 = ethidium homodimer-1; PBS= phosphate-buffered saline.

Figure 4: Representative safranin O staining of the femoral condyle in the loaded knee. A slide showing the sagittal sections of the lateral femoral condyle, which were stained with a safranin O/Fast Green and hematoxylin solution. Compared to the control, the safranin O staining intensity in the affected area was decreased after loading, and a clear border (arrow) of the

upper/calcified cartilage was observed. Scale bars = 100 µm. Abbreviation: w = week.

DISCUSSION:

For the first time, the current protocol shows how to establish a model of loading-induced cartilage lesion on the lateral femoral condyle in rats, similar to the intra-articular damage model in smaller rodents such as the mouse[2]. However, the loading protocol in mice caused severe osteophyte formation and cruciate ligament lesions, which was not ideal for evaluating the effects of cyclic compression. The current protocol created a focal cartilage lesion in rats with a much lower loading force. Correct loading method settings are critical for the protocol because only the appropriate magnitude, speed, and duration of stress can destroy the cartilage without damaging the bone tissue.

Setting the displacement limit (protocol step 1.3.1) is also crucial as it immediately stops the instrument in case of ligament rupture or if the rat wakes up from anesthesia during the loading session. The optimal maximum load and the age of the rat remain to be determined. However, in the preliminary experiments, a load of over 50 N resulted in a high probability of ACL rupture in the rat's knees. Moreover, the current model is difficult to reproduce in older (>36 weeks old) rats, possibly due to the stiffness of the cartilage as growth occurs.

Although the destructive load threshold for younger rats was not determined, it is believed that future studies should keep the maximum load under 20 N to observe any anabolic effects on the cartilage. The scope and localization of the lesion area were relatively straightforward to establish, even for those new to the field, as estimated by the chondrocyte-degenerative volume in each sample, which potentially focused on the subsequent evaluation of the intervention to a relatively narrow cartilage area.

Histological staining demonstrated that the scope of the lesion area was relatively steady during the 8-week observation period. However, Mankin's scores deteriorated continuously while the matrix staining and cell distribution scores increased in the affected area. Moreover, there was an obvious color deviation between the middle layer and the calcified cartilage, which illustrated that only the cartilage above the tidemark was affected by the interarticular compression.

On the contrary, apart from mild fibrillation in rare samples, the integrity of the cartilage remained largely intact throughout the entire observational period, which is different from progressive OA injury models[10]. Therefore, a non-surgical model may be better for the assessment of cartilage interface collision-induced focal lesions, which are more common in sports injuries. In the future, the current model will be used to assess the effects of medication or physical therapy, such as hyperthermia therapy and aerobic joint exercise, on traumatic cartilage damage. Moreover, chondrocyte anabolism and catabolism in response to cyclic mechanical stimulation could also be validated *in vivo* in animals using this model.

The current protocol had several limitations. First, only cartilage lesions on the lateral femoral condyle were investigated. The lesion on the lateral tibia should also be evaluated in future studies. Second, the lesioned part of the articular cartilage studied in the current protocol was

not the main loading-bearing region during walking. Due to the heterogeneity of cartilage, the stiffness of the intra-articular cartilage may differ from the part examined in the current study. Thus, these findings can only be used as a reference. Finally, the model did not show any significant progression of cartilage degeneration, which is an important feature of OA development. Further studies could combine invasive surgery with pre-loaded lesions to observe spatiotemporal changes.

ACKNOWLEDGMENTS:

This study was supported in part by a JSPS KAKENHI grant (numbers JP18H03129 and JP18K19739).

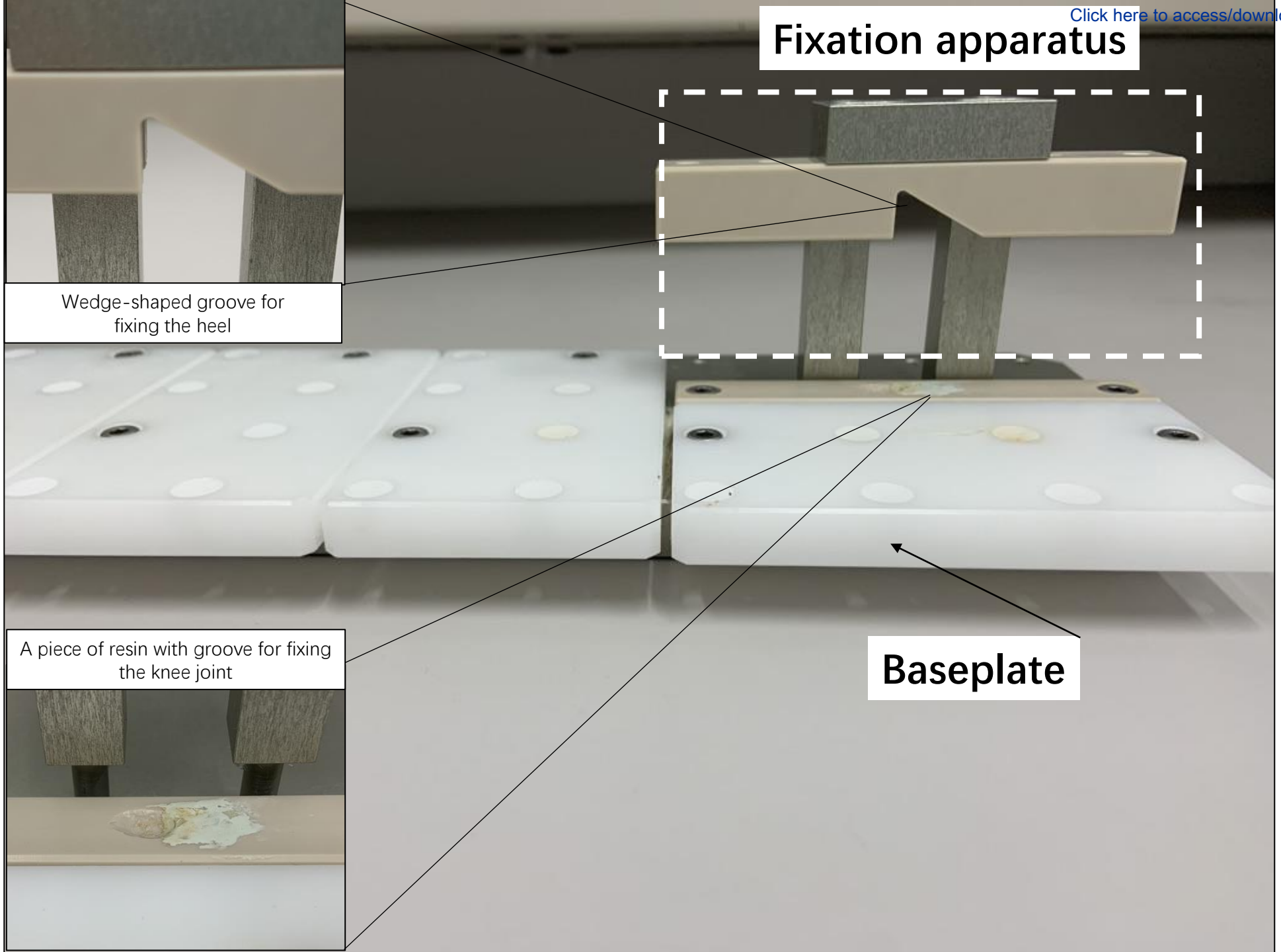
DISCLOSURES:

The authors declare no conflicts of interest.

REFERENCES:

1. De Souza, R. L. et al. Non-invasive axial loading of mouse tibiae increases cortical bone formation and modifies trabecular organization: a new model to study cortical and cancellous compartments in a single loaded element. *Bone.* **37** (6), 810–818 (2005).
2. Poulet, B., Hamilton, R. W., Shefelbine, S., Pitsillides, A. A. Characterizing a novel and adjustable noninvasive murine joint loading model. *Arthritis and Rheumatism.* **63** (1), 137–147 (2011).
3. Wu, P. et al. Early response of mouse joint tissue to noninvasive knee injury suggests treatment targets. *Arthritis and Rheumatism*. **66** (5), 1256–1265 (2014).
4. Poulet, B. et al. Intermittent applied mechanical loading induces subchondral bone thickening that may be intensified locally by contiguous articular cartilage lesions. *Osteoarthritis Cartilage.* **23** (6), 940–948 (2015).
5. Ko, F. C. et al. Progressive cell-mediated changes in articular cartilage and bone in mice are initiated by a single session of controlled cyclic compressive loading. *Journal of Orthopaedic Research.* **34** (11), 1941–1949 (2016).
6. Adebayo, O. O. et al. Role of subchondral bone properties and changes in development of load-induced osteoarthritis in mice. *Osteoarthritis Cartilage.* **25** (12), 2108–2118 (2017).
7. Ko, F. C. et al. In vivo cyclic compression causes cartilage degeneration and subchondral bone changes in mouse tibiae. *Arthritis and Rheumatism.* **65** (6), 1569–1578 (2013).
8. Ji, X. et al. Effects of in vivo cyclic compressive loading on the distribution of local Col2 and superficial lubricin in rat knee cartilage. *Journal of Orthopaedic Research.* **39** (3), 543–552 (2021).
9. Kawai, S., Takagi, Y., Kaneko, S., Kurosawa, T. Effect of three types of mixed anesthetic agents alternate to ketamine in mice. *Experimental Animals*. **60** (5), 481-487 (2011).
10. Iijima, H. et al. Destabilization of the medial meniscus leads to subchondral bone defects and site-specific cartilage degeneration in an experimental rat model. *Osteoarthritis Cartilage.* **22** (7), 1036–1043 (2014).

Figure 1



Figure

Click here to access/download;Figure;Figure 2 (1).pdf

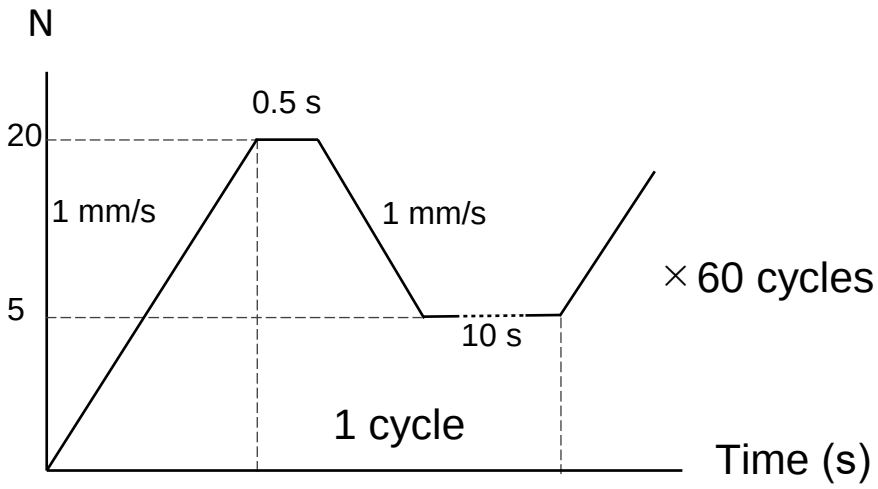
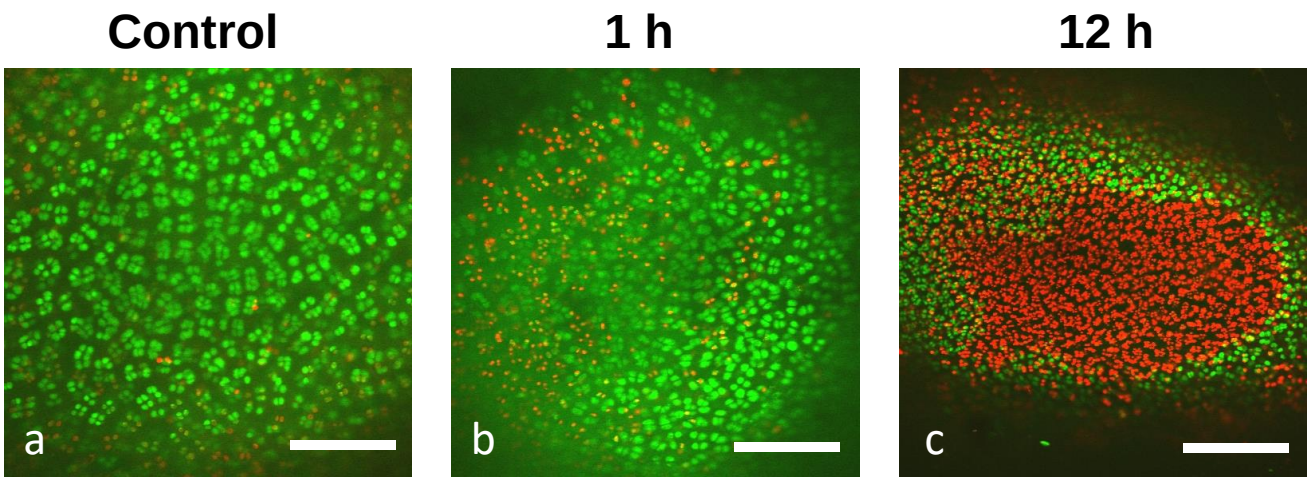
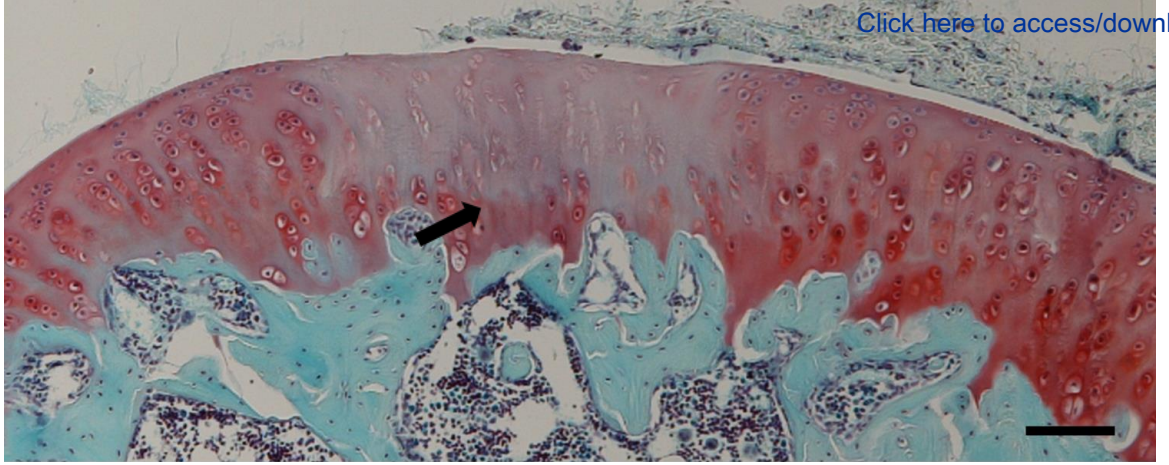


Figure 3



Figure

2 w



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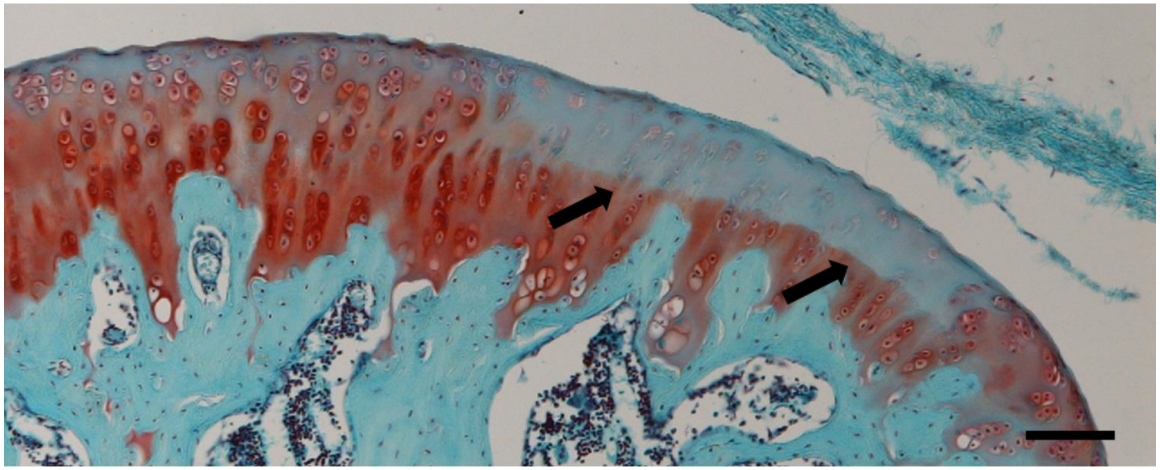
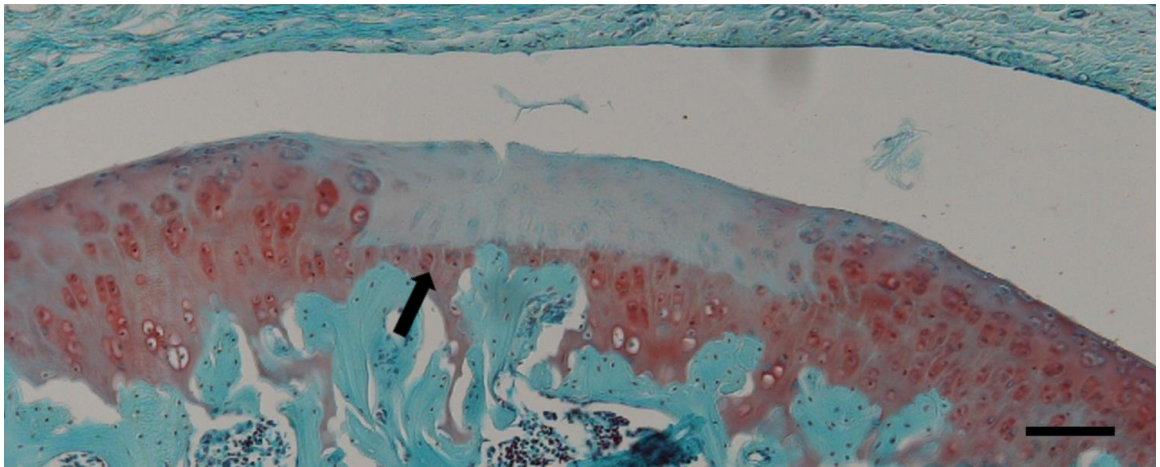


8 w



Name of Material/Equipment	Company	Catalog Number
Anesthetic Apparatus for Small Animals	SHINANO MFG CO.,LTD.	SN-487-0T
Autograph AG-X	Shimadzu Corp	N.A.
Fluoview FV10i microscope	Olympus Corp	N.A.
ISOFLURANE Inhalation Solution	Pfizer Japan Inc.	(01)14987114133400
LIVE/DEA Viability/Cytotoxicity Kit	Thermo Fisher Scientific Japan Inc	L3224
TRAPEZIUM X Software	Shimadzu Corp	N.A.

Comments/Description

Precision Universal / Tensile Tester
A fully automated confocal laser-scanning microscope

A quick and easy two-color assay to determine viability of cells
Data processing software for Autograph AG-X

May 20, 2021

Dear Dr. Amit Krishnan

Review Editor

JoVE

Manuscript ID: **JoVE62660**

Title (revised)

"**A non-invasive method for generating the cyclic loading-induced intra-articular cartilage lesion model of the rat knee**"

[Information in advance]

Thank you for the helpful comments and suggestions. New **References** & **Figures** is added based on Editor & Reviewer's advice. The order of **References** & **Figures** is rearranged. Our actions in response to Editor & Reviewers comments are described below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

[Authors' response]

Thank you for the comments. We've reedited the English proofreading through Editage service. Please see the attached file of **Certificate_of_editing**.

2. Please revise the following lines to avoid previously published work: 28-29, 31-33, 114-115, 160-161.

[Authors' response]

We rewrote these sentences you mentioned, please see line 29-34, 132-133, 189-190 of **manuscript**.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

[Authors' response]

Thank you for the reminder. All the personal pronouns are now revised.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

For example: Autograph AG-X, TRAPEZIUM X Software, LIVE/DEAD Viability/Cytotoxicity Kit, Thermo Fisher Scientific, Fluoview FV10i, Olympus, etc.

[Authors' response]

Thank you for the reminder. All the commercial languages are now removed.

5. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

[Authors' response]

The **Introduction** section is now revised. For the requirement a) & e), please see line 57-60. For the requirement b), please see line 49-50. For the requirement c), please see line 50-55. For the requirement d), please see line 44-48.

6. Line 69-71: Please mention how proper anesthetization is confirmed.

[Authors' response]

We added the contents in Protocol 1.1.2 section, please see line 75-78.

7. Line 75-78: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

8. Line 76: Please include the dimensions of the resin.

[Authors' response]

Thank you for the crucial reminder. We've substantially revised the whole paragraph of 1.2.1. We also added a new figure of Figure 1 in order to interpretate the "frame" more clearly, please see the new attached file of **Figure 1** & line 82-85.

9. Line 80-81: Please mention how is the calibration performed.

[Authors' response]

We added sentences in the Protocol 1.2.2 section, please see line 87-89.

10. Line 86-98: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

[Authors' response]

Thanks for the crucial judgement. We have rewritten this section completely, please see line 96-117.

11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

[Authors' response]

We revised and added some contents to meet the requirements. For the requirement a), please see line 174-178. For the requirement b), please see line 178-184. For the requirement c), please see line 203-211. For the requirement d), please see line 169-174. For the requirement e), please see line 198-201.

12. Please consider adding more references to the manuscript. The minimum number of references required is 10.

[Authors' response]

Thank you for the reminder. We now added references to meet the requirements, please see the **References** section (line 221-255).

13. Figure 1: Please include scale bars in all the figures in the panel. Define the scale bars in the Figure or the Figure Legends. Consider providing the details of magnification in the Figure Legends.

[Authors' response]

Thank you for the reminder. Scale bar in Figure and legends are now added, please see the attached file of **Figure 3** & sentences in line 160.

14. Please ensure that the Table of Materials includes all the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

[Authors' response]

We added some information in Table of Materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This protocol establishes a method for creating a femoral condylar defect on a rat knee.

Major Concerns:

This protocol is very specific to the exact equipment used, particularly the Autograph AG-X and TRAPEZIUM X Software. There is value in having a very specific protocol so that the experiment could be reproduced exactly. However if the authors indicate the reason for some of the steps of the protocol, it would be helpful so that others could adapt it to their specific equipment. Otherwise, this protocol is too specific and will limit the audience.

[Authors' response]

Thank you for your helpful comments. Theoretically, any controllable loading instrument with a flat facet of load cell could reproduce our protocol. However, the accuracy may diversed from each manufacturer brand. Therefore, we added reasons for some specific steps to make sure the experiment could be reproduced in other instruments. Please see the modification in **Protocol** section line 91-92, 99-100, 106-108, and 112-113.

Minor Concerns:

1.2.1. Why do you use isoflurane and the cocktail of injected anesthetics? Why the cocktail instead of one? or just isoflurane?

[Authors' response]

There reason we did not apply inhalation anesthesia for the whole period of loading operation is that we have already confirmed some death cases in experimental rats when they exposed in atomized isoflurane longer than 20 min. Although we did not specify the reason, we decided not to use long time inhalation anesthesia alone for any types of traumatic operation. The cocktail was recommended by the Kyoto University Regulations for the Animal Experiment Committee, which is based on the published article by Kawai S. et al[1]. It was said the Antagonism of mixture could prevent animals from anesthesia excess state. We enclosed this reference to the main text as well.

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1. Kawai et al., Exp. Anim., 481-487, 2011

1.2.1 "face down" is strange wording for a rat as even when they are on their belly their face is up. Rephase to indicate laying on their back or belly.

[Authors' response]

Thanks for the reminder. We revised the sentence in line 82.

1.2.1. This step of the protocol is unclear. Perhaps with a video it is more clear but it seems like it is some type of frame which fixes the knee and holds the ankle. Unclear what the notch is, why 140 deg? What is the important function of this step: hold the knee in some sort of cup, flex the knee, "fix the ankle" - is this also in a cup? Fine to keep it very specific to your set up but make sure we know what is happening so that people could adapt to their own set-up.

[Authors' response]

Thank you for the crucial reminder. We've substantially revised the whole paragraph of 1.2.1. We also added a new figure of Figure 1 in order to interpretate the "frame" more clearly, please see the new attached file of **Figure 1** & line 82-85.

1.3.1 This is extremely detailed for the specific TRAPEXZIUM software. Drawing a load profile, or indicating in a graph what the load profile is would be much easier to adapt to a different machine.

[Authors' response]

We inserted a line chart of load profile in the section, please see the new **Figure 2**.

1.3.1 The 20N load protocol is for 12 week old rats. It would be helpful to put the weight of the rats so that size could be somewhat controlled.

[Authors' response]

The average weight of Wistar rats at 12-week-old was 256.8g. We added this information in the **Protocol** section (line 72).

It is unclear if Figure 1 will be part of the video. If so, put scale bar (they look different scales) and use control images at the same time points.

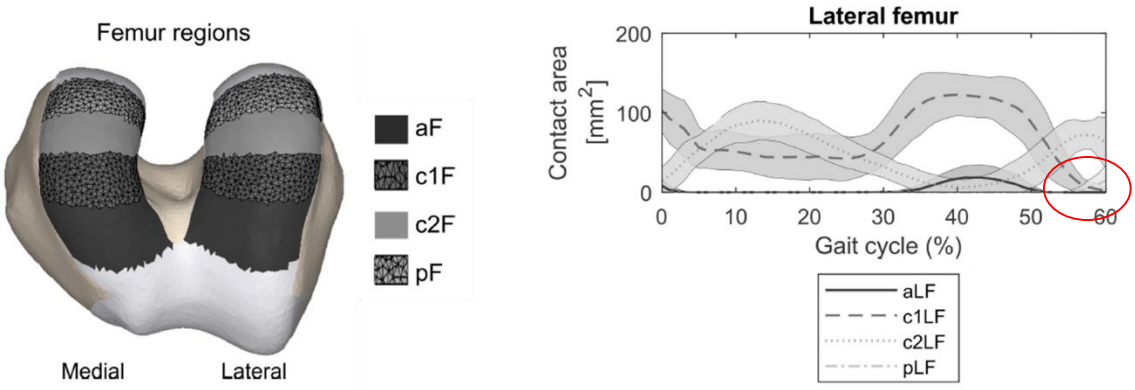
[Authors' response]

Sorry for the equivocal description. The images of Figure 1 was taken in three separate samples of control, 1h after loading and 12 h after loading. The control image was taken at 1 hour after 12min preload. Tiny differences of scales may due to the different depth of fluorescent microscope scanned images. We added the scale bar on the Figure 3, please see attached file of **Figure 3**.

Line 170: Discussion: "the lesion part of the articular cartilage in the current protocol did not play the main role of loading during walking" What does this mean?

[Authors' response]

As a published research[1] demonstrated (see the figure below), in human, the posterior part of lateral femur cartilage contact with its counterpart during only a small portion of gait stance phase of gait. It was also reported that cartilage is heterogeneous, there is an enormous variation in the stiffness of cartilage in different location. we added some more information in the limitation section, please see line 205-207.



Refrence figure: Cartilage contact area for posterior femur regions throughout the stance phase of gait. There were a little portion during the end of stance phase (red circle), posterior lateral femur have contact with its counterpart. (quoted from Zevenbergen L, et al, PLoS One, 2018[1], the original data of Figure 1A [left panel] and Figure 2A [right panel])

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1. Zevenbergen L, et al. Cartilage defect location and stiffness predispose the tibiofemoral joint to aberrant loading conditions during stance phase of gait. PloS ONE. 2018;13(10):e0205842

Line 173: "progression of cartilage degeneration" Mouse studies have shown that one day of loading creates a lesion that does not progress, continued loading for a week will serve to progress the lesion. Did you check this? Should you mention this?

[Authors' response]

I thought you're mentioning the studies by Poulet B (Reference #2), which found multiple session of loading could induce the worsen of cartilage lesion severity. However, it reported a cruciate ligament lesion and osteophytes formation for nearly all multiple sessions samples but not in one day loading samples. We considered the osteophytes could unbalance the homeostasis of bone metabolism, and more importantly, anterior cruciate injury could be the direct factors of PTOA. Therefore, we thought the recipe used in previous research was a little confused and maybe overdose. We added this information in the **Discussion** section, please see. Line 171-172.

Reviewer #2:

Manuscript Summary:

The manuscript by Xiang Ji et al. reports a non-invasive method of cyclic loading induced intra-articular lesion model on rat knee. In this study, the authors tried to develop a non-surgical posttraumatic osteoarthritis (PTOA) model by in vivo cyclic compressive loading to avoid surgery-induced intra-articular inflammation. This is an interesting approach to the development of joint injury. However, the authors' statement in the Abstract that this is a novel PTOA rat model is not supported by the experimental results.

Major Concerns:

Although cellular analysis showed chondrocyte death after the cyclic loading, the histologic changes are limited to focalized loss of safranin-O staining with little or no osteoarthritic structural alterations. It would be appropriate to describe the presented histologic changes as focal cartilage degeneration or dysfunction rather than PTOA because previous studies have demonstrated that loss of proteoglycans in cartilage could be fully reversible.

The authors' statement in the Abstract "we describe the experimental protocol of this novel PTOA rat model" should be modified carefully to reflect the experimental results.

[Authors' response]

Thank you for the crucial reminder. The Abstract portion is now revised, please see line 28-41.

Minor Concerns:

The nature of "intra-articular lesion" in the title should be specified.

[Authors' response]

Thank you for the reminder. The title of this paper is now revised.

We would like to thank the editor & reviewers for the helpful comments and hope that the revised manuscript is acceptable for publication in ***JoVE***.

Sincerely

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MANUSCRIPT TITLE

A non-invasive method for generating the cyclic loading-induced intra-articular cartilage lesion model of the rat knee

AUTHORS

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